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Immobilized ferredoxins for affinity chromatography of ferredoxin-dependent enzymes

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ABSTRACT

An immobilized ferredoxin more stable than the conventional immobilized spinach ferrodoxin was prepared by reacting CNBr-Sepharose with ferredoxins isolated from barley and *Synechococcus vulcanus*, a thermophilic blue-green alga. The dissociation constants of immobilized ferredoxin from spinach, barley and *S. vulcanus* for spinach ferredoxin–NADP reductase were 0.922, 2.505 and 5.209 μM, respectively, whereas those for barley ferredoxin–NADP reductase were 1.159, 0.579 and 2.851 μM, respectively. The order of stability was *S. vulcanus* > barley > spinach. The immobilized ferredoxin was applied to the simultaneous detection of ferredoxin-dependent enzymes in spinach chloroplasts. Over 20 polypeptides were detected. *Synechococcus* ferredoxin could also be immobilized on a Toyopearl gel and repeatedly used in an automated high-performance liquid chromatographic system.

INTRODUCTION

In 1968, it was found that ferredoxin conjugated ferredoxin–NADP reductase (FNR) to form a stable protein–protein complex under low salt conditions and released it under high salt conditions [1–4]. These investigations provided the background for subsequent studies on affinity chromatography using immobilized ferredoxins. Sugiyama and Yamano [5] immobilized bovine adrenal ferredoxin (adrenodoxin) on Sepharose gel and used it for affinity chromatography of adrenodoxin reductase (adrenal FNR). Shin and Oshino [6] immobilized spinach ferredoxin on Sepharose gel and introduced it into the purification method for spinach FNR.

In chloroplasts, several ferredoxin (Fd)-dependent enzymes such as nitrite reductase [7], sulphite reductase [8], glutamate synthase [9] and thioredoxin reductase [10] are present in addition to FNR. As all these enzymes are capable of forming complexes with ferredoxin [11], the immobilized ferredoxin has grown to be an indispensable tool for the isolation and purification of these enzymes. However, application of the immobilized ferredoxin has been limited so far to the isolation and purification of individual Fd-dependent enzymes. Here we attempted to expand the application of this affinity technique to the simultaneous detection of Fd-dependent enzymes in plants.

Spinach ferredoxin has been most popularly immobilized on Sepharose 4B as an affinity ligand. However, we have recognized that the spinach ferredoxin gradually lost its ability to bind FNR on the Sepharose gel during repeated use, in parallel with the loss of its red colour characteristic of an iron–sulphur centre. A more stable ferredoxin ligand might be required for immobilization in order to overcome this problem. We therefore examined the heat stability of ferredoxins prepared from several plants and chose two candidates from barley and *Synechococcus vulcanus*, a thermophilic blue-

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green alga. The properties of the new ferredoxin-Sepharose (Fd-Sepharose) thus prepared were compared with the conventional spinach Fd-Sepharose with respect to binding affinity towards FNR and stability.

The Sepharose gel matrix is not mechanically stable enough to endure accelerated chromatographic operation in an automated chromatographic system. We therefore attempted to immobilize *Synechococcus* ferredoxin on Toyopearl gel, a hydrophilic vinyl polymer gel matrix that is mechanically much more stable than Sepharose gel. The *Synechococcus* Fd-Toyopearl was successfully used repeatedly for the purification of spinach FNR in an automated preparative chromatographic system.

EXPERIMENTAL

Materials

CNBr-Sepharose 4B was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). AF-Tresyl Toyopearl 650M and other TSK gels were obtained from Tosoh (Tokyo, Japan).

Ferredoxin preparations

Ferredoxins from spinach, barley and wheat were prepared essentially as described by Sakihama *et al.* [12]. Ferredoxins from *Synechococcus vulcanus*, *Mastigocladus laminosus*, thermophilic blue-green algae and *Spirulina platensis*, a non-thermophilic blue-green alga, was isolated according to Shin *et al.* [13].

All ferredoxins obtained above were determined by using a molar absorptivity of 9.68 l $mmol^{-1}$ cm^{-1} at 420 nm [14] and a relative molecular mass (M_r) of 11 500 [15] reported for spinach ferredoxin.

Immobilization of ferredoxin

Fd-Sepharose. Ferredoxins from spinach, barley and *S. vulcanus* were immobilized on Sepharose 4B by reacting individual ferredoxin with CNBr-Sepharose 4B according to the reported procedure [6].

Fd-Toyopearl. Synechococcus ferredoxin (10 mg) was dissolved in 2 ml of the coupling buffer [0.1 *M* $NaHCO_3$ (pH 8.0) containing 0.5 *M* NaCl]. To this ferredoxin solution, 0.2 g of AF-Tresyl Toyopearl 650M were added and the mixture was shaken at 25°C for 24 h. Then the mixture was transferred to a glass filter and the mother solution was removed. The red Toyopearl gels on the glass sieve were washed well with coupling buffer and suspended in blocking buffer [0.1 *M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* NaCl] to inactivate unreacted tresyl groups. Finally, it was washed with stock buffer solution [20 m*M* Tris–HCl buffer (pH 7.5) containing 0.5 *M* NaCl] and stored in that buffer at 4°C. A 0.2-g amount of dry Tresyl-Toyopearl yielded about 1 ml of *Synechococcus* Fd-Toyopearl.

Measurement of heat stability of ferredoxins

Purified ferredoxins were incubated in 20 m*M* Tris–HCl buffer (pH 7.5) at 45°C and the absorbance at 420 nm was measured at 5-min intervals.

Determination of dissociation constant of immobilized ferredoxin for FNR

The dissociation constant of immobilized ferredoxin for FNR was determined by essentially the same method as described previously [16]. Immobilized Fd, 0.2-ml packed volume, was suspended with 20 m*M* Tris–HCl buffer (pH 7.5) in a small spectrophotometric cell with a 2-ml total volume. A concentrated solution of FNR dissolved in the same buffer was added to the suspension with mixing for reaction with the immobilized ferredoxin. After the cell was centrifuged at 270 *g* for 5 min in a bench-top centrifuge (Microlabofuge, Model M-15, Sakuma, Tokyo, Japan) to sediment the immobilized ferredoxin, the absorbance of the supernatant was directly measured to determine free and bound FNR.

The dissociation constant, K_d, was calculated based on the following equation:

$$\frac{[\mathrm{FNR}]_{\mathrm{free}}}{[\mathrm{FNR}]_{\mathrm{total}} - [\mathrm{FNR}]_{\mathrm{free}}} = \frac{K_d + [\mathrm{FNR}]_{\mathrm{free}}}{[\mathrm{Fd}]_{\mathrm{bound}}} \qquad (1)$$

where $[\mathrm{FNR}]_{\mathrm{free}}$ is the concentration of free FNR remaining in the supernatant, $[\mathrm{FNR}]_{total}$ that of FNR added and $[\mathrm{Fd}]_{\mathrm{bound}}$ that of immobilized ferredoxin in the reaction mixture that is active in binding FNR.

Determination of amount of active ferredoxin

The amount of active bound ferredoxin capable of binding spinach FNR on the gel matrix was determined from $[\mathrm{Fd}]_{\mathrm{bound}}$ in eqn. 1 according to the following equation:

Active ferredoxin on gel matrix (nmol ml^{-1} wet gel) =

$$[Fd]_{bound} \cdot \frac{\text{total volume of reaction mixture (ml)}}{\text{packed volume of immobilized Fd (ml)}} \quad (2)$$

$[Fd]_{bound}$ can be calculated by plotting $[FNR]_{free}/([FNR]_{total} - [FNR]_{free})$ against $[FNR]_{free}$ and taking the reciprocal of the gradient of the straight line.

FNR preparation

FNR was prepared from spinach and barley essentially as described by Sakihama *et al.* [12]. Its amount was determined by using a molar absorptivity of 10.74 l $mmol^{-1}$ cm^{-1} at 460 nm [17]. The enzymatic activity of FNR was assayed in terms of NADPH–diaphorase activity as described previously [18].

Simultaneous detection of Fd-dependent enzymes in spinach chloroplasts

Extraction. Fd-dependent enzymes were extracted from spinach chloroplasts as follows: 150 g of spinach leaves were homogenized in 450 ml of an isotonic medium containing 10 m*M* sodium pyrophosphate–HCl buffer (pH 6.5), 0.35 *M* sucrose, 4 m*M* $MgCl_2$ and 2 m*M* sodium ascorbate [19] for 30 s in a mixer. After the homogenate had been filtered using a basket-type centrifuge (N-110N, Kokusan Enshinki, Tokyo, Japan), the filtrate was centrifuged at 1000 *g* for 7 min to sediment chloroplasts. The chloroplasts were resuspended in 45 ml of the isotonic medium and centrifuged at 1000 *g* for 10 min. The washed chloroplasts were suspended in 30 ml of 50 m*M* Tris–HCl buffer (pH 7.9) containing 5 m*M* EDTA and kept on ice for 30 min. The supernatant was collected by centrifugation at 20 000 *g* for 20 min.

Adsorption on ferredoxin column. The chloroplast extract was passed through a Sephadex G-25 column (30 × 5 cm I.D.) equilibrated with 10 m*M* Tris–HCl buffer (pH 7.9) to remove contaminating low-molecular-mass substances. Fractions eluted at the void volume were loaded on an Fd-Sepharose column (4 × 2 cm I.D.), prepared with spinach or *Synechococcus* ferredoxin, which had been equilibrated with the same buffer. After washing the column with three column volumes of the same buffer, Fd-dependent enzymes were eluted with 0.1 *M* Tris–HCl buffer (pH 7.5) containing 1 *M* NaCl. The elution was monitored by measuring the absorbance at 280 nm and the NADPH–diaphorase activity of the eluate. The active fractions were combined and concentrated by ultrafiltration using Centriflo CF-25 (Amicon, Danvers, MA, USA). After solvent exchange to 10 m*M* Tris–HCl buffer (pH 7.9) by repeated ultrafiltration, the volume of the enzyme sample was adjusted to 0.5 ml.

SDS-PAGE. The resolving gel contained an acrylamide concentration gradient (7.5–15%) and the stacking gel contained 6% acrylamide. Electrophoresis was carried out at a constant current of 30 mA and polypeptide bands were revealed by the method of Fish and Jagendorf [20]. Relative molecular mass (M_r) markers used were bovine serum albumin (67 000), ovalbumin (45 000), spinach FNR (33 000), α-chymotrypsinogen A (25 000) and myoglobin (17 500).

Operation of affinity column chromatography

Barley Fd-Sepharose column. The immobilized barley ferredoxin on Sepharose 4B was packed in a glass column (10 × 1 cm I.D.) to a height of 4 cm. After the barley Fd-Sepharose column (4 × 1 cm I.D.) had been equilibrated with 20 m*M* Tris–HCl buffer (pH 7.5), about 100 nmol of partially purified spinach FNR dissolved in 2 ml of the same buffer were placed on the column. The column was washed with 5 ml of the same buffer and the adsorbed FNR was eluted with 0.1 *M* Tris–HCl buffer (pH 7.5) containing 0.25 *M* NaCl. When the chromatography was finished, the column was washed well with 0.1 *M* Tris–HCl buffer (pH 7.5) containing 0.5 *M* NaCl and stored at 4°C.

Synechococcus Fd-Toyopearl column. The immobilized *Synechococcus* ferredoxin on Toyopearl 650M was packed in a pressure-resistant glass column (10 × 2.2 cm I.D., C.I.G. type, Kusano Kagakukikai, Tokyo, Japan) to a height of 4.1 cm and placed in a Toyopearl Chromato fast-flow liquid chromatographic apparatus (Tosoh, Tokyo, Japan). The column was automatically equilibrated with 45 ml of 20 m*M* Tris–HCl buffer (pH 7.5), loaded with a crude FNR preparation dissolved in the same buffer, which was obtained from 2 kg of spinach leaves, washed with 45 ml of the same buffer and eluted with 35 ml of 20 m*M* Tris–HCl buffer (pH 7.5) containing 0.25 *M* NaCl at a flow-rate of

1 ml min^{-1}. After completion of the chromatography, the column was washed well with 20 m*M* Tris–HCl buffer (pH 7.5) containing 0.5 *M* NaCl and stored at 4°C.

RESULTS AND DISCUSSION

Heat stability of ferredoxins

Ferredoxins were isolated and purified from three species of higher plants, spinach, wheat and barley, two thermophilic blue-green algae, *S. vulcanus* and *M. laminosus*, and a non-thermophilic blue-green alga, *S. platensis*. These purified ferredoxins were examined for heat stability at 45°C in a low-salt buffer at pH 7.5 as shown in Fig. 1. The order of heat stability among the six ferredoxins was found to be *S. vulcanus* > *M. laminosus* > barley > *S. platensis* > wheat > spinach. Ferredoxins from blue-green algae tend to be more stable than ferredoxins from higher plants. Also, ferredoxins from thermophilic plants were more stable than those from non-thermophilic plants.

As *Synechococcus* ferredoxin was the most stable of the six ferredoxins examined and barley ferredoxin was the most stable of the three higher plant ferredoxins, these two ferredoxins were chosen for immobilization.

Properties of immobilized ferredoxins on Sepharose

Coupling reactions of the two ferredoxins from *S. vulcanus* and barley with CNBr-Sepharose 4B were successfully achieved in the same manner as spinach ferredoxin [6]. The properties of the two immobilized ferredoxins are summarized in Table I.

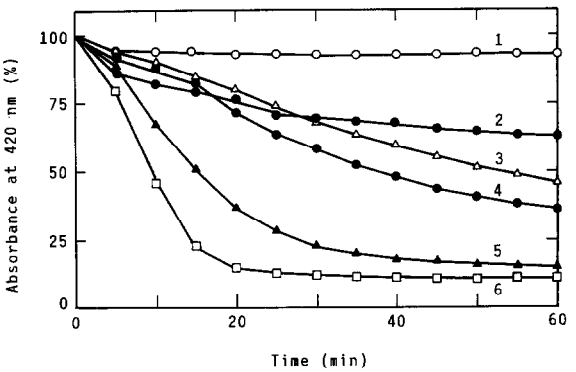

Fig. 1. Heat stability of ferredoxins. Ferredoxins were incubated in 20 m*M* Tris–HCl buffer (pH 7.5) at 45°C. Absorbance at 420 nm was recorded at 5-min intervals. 1 = *S. vulcanus*; 2 = *M. laminosus*; 3 = barley; 4 = *S. platensis*; 5 = wheat; 6 = spinach.

The dissociation constants, K_d, of the three immobilized ferredoxins from spinach, barley and *Synechococcus* for spinach FNR were calculated to be 0.922, 2.505 and 5.209 μM, respectively, and those for barley FNR were 1.159, 0.579 and 2.851 μM, respectively. These results imply that binding between ferredoxin and FNR is more specific when the origins of the two proteins are phylogenetically closer. Recently, Ida *et al.* [21] used a barley Fd-Sepharose column for the purification of nitrite reductase from rice because the affinity of a spinach Fd-Sepharose column toward the rice enzyme was not high enough to give a clear separation of enzyme from impurities.

Spinach, barley and *Synechococcus* Fd-Sepharose contained at first 70.4, 58.1 and 97.9 nmol ml^{-1} wet

TABLE I

PROPERTIES OF IMMOBILIZED FERREDOXINS FROM SPINACH, BARLEY AND *S. VULCANUS*

Parameter	Sepharose 4B gel matrix			Toyopearl 650M gel matrix:
	Spinach	Barley	*S. vulcanus*	*S. vulcanus*
K_d (μM) for spinach FNR	0.922	2.505	5.209	1.543
K_d (μM) for barley FNR	1.159	0.579	2.851	–
Active ferredoxin[a] (nmol ml^{-1} wet gel)	70.4	58.1	97.9	34.4
Stability[b]	+	++	++++	++++

[a] Ferredoxin capable of binding spinach FNR.
[b] Judged from red colour remaining after affinity titration had been repeated five times.

gel of active ferredoxin capable of binding spinach FNR, respectively. When affinity titration had been repeated five times, the red colour of spinach Fd-Sepharose was severely diminished, whereas that on *Synechococcus* Fd-Sepharose remained almost unaffected. Barley Fd-Sepharose showed an intermediate stability. Hence the order of stability among these three ferredoxin ligands on Sepharose was consistent with that among soluble ferredoxins.

Preparation of Fd-Toyopearl

Despite *Synechococcus* ferredoxin having the least specificity for spinach FNR on Sepharose, its outstanding stability was attractive considering the introduction of an automated fast-flow liquid chromatographic system into the Fd-affinity chromatography. Therefore, *Synechococcus* ferredoxin was reacted with tresyl-activated Toyopearl 650M. The properties of *Synechococcus* Fd-Toyopearl are shown in Table I. K_d for spinach FNR was calculated to be 1.543 μM, which is considerably lower than that of the ferredoxin immobilized on Sepharose. It contained 34.4 nmol ml^{-1} wet gel of active ferredoxin for spinach FNR.

Application to simultaneous detection of Fd-dependent enzymes

So far, five distinct Fd-dependent enzymes have been isolated from spinach leaves as listed in Table II. As all these enzymes share electrons from one common donor, ferredoxin, *in vivo*, it is of interest to investigate how these enzymes are related to each other under the physiological conditions. We therefore attempted to gather all Fd-dependent enzymes present in spinach choroplast by adsorption on a ferredoxin column and identify them by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) based on their molecular mass as indicated in Table II. As shown in lane 1 in Fig. 2, over 20 polypeptide bands with M_r ranging from 13 000 to 112 000 were detected. Although the polypeptide with M_r = 35 000 could be attributed to FNR with the aid of an immunoblotting method, it was difficult to distinguish those of known Fd-dependent enzymes other than FNR by simply comparing their molecular masses. Further investigations are needed for the identification of individual polypeptides. The number of polypeptides that appeared on the gel was by far greater than that expected to be generated from five Fd-dependent enzymes, suggesting possibility that there are new Fd-dependent enzymes in chloroplasts.

When a *Synechococcus* Fd-Sepharose column was used in place of a spinach Fd-Sepharose column, an almost identical electrophoretic pattern of polypeptides was obtained as shown in lane 2 in Fig. 2. All polypeptide bands detected in lane 1 are present also in lane 2, but some polypeptide bands were slightly diminished. Although the binding specificity of the *Synechococcus* Fd-Sepharose column was not exactly identical with that of the spinach Fd-Sepharose column, both columns can be used equiv-

TABLE II

PROPERTIES OF FERREDOXIN-DEPENDENT ENZYMES FROM SPINACH

Enzyme	Reaction[a]	Active centre(s)	M_r	Ref.
NADP reductase	$2Fd_{red} + NADP^+ + 2H^+ \rightarrow 2Fd_{ox} + NADPH + H^+$	FAD	35 000	17, 18
Nitrite reductase	$6Fd_{red} + NO_2^- + 8H^+ \rightarrow 6Fd_{ox} + NH_4^+ + 2H_2O$	Sirohaeme Fe_4S_4	63 000	22
Sulphite reductase	$6Fd_{red} + SO_3^{2-} + 6H^+ \rightarrow 6Fd_{ox} + S^{2-} + 3H_2O$	Sirohaeme Fe_4S_4	69 000 × 2	23
Glutamate synthase	$2Fd_{red}$ + glutamine + 2-oxoglutarate $\rightarrow 2Fd_{ox}$ + 2glutamate	Fe_2S_2 × 2 FAD, FMN	170 000	24, 25
Thioredoxin reductase	$2Fd_{red} + Td_{ox} + 2H^+ \rightarrow 2F_{ox} + Td_{red}$	Fe_4S_4 –S–S–	13 000 + 16 000	26

[a] Fd, ferredoxin; Td, thioredoxin.

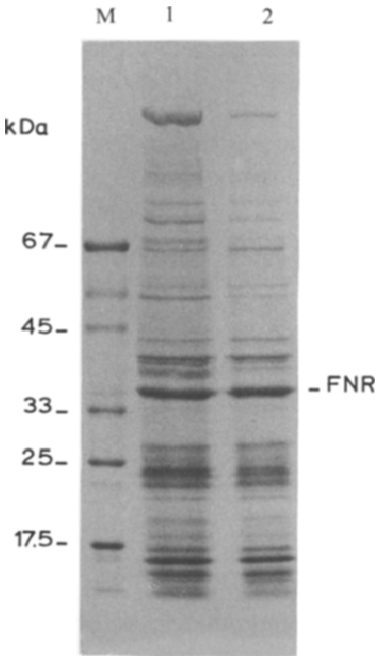

Fig. 2. Simultaneous detection of Fd-dependent enzymes in spinach chloroplasts. Fd-dependent enzymes were extracted from spinach chloroplasts and adsorbed on a spinach Fd-Sepharose column. The eluate from the column was analysed by SDS-PAGE in lane 1. Fd-dependent enzymes adsorbed on a *Synechococcus* Fd-Sepharose column were analysed in lane 2. Lane M = M_r markers (kDa = kilodalton): bovine serum albumin (67), ovalbumin (45), spinach FNR (33), α-chymotrypsinogen A (25) and myoglobin (17.5).

alently for the detection of Fd-dependent enzymes. Both the binding capacity and the red colour of the *Synechococcus* Fd-Sepharose column remained essentially unchanged during repeated adsorption of chloroplast extracts, whereas a considerable decrease in both binding capacity and red colour was observed with a spinach Fd-Sepharose column even after a single use under the same experimental conditions.

Application to column chromatography

Fig. 3 shows a chromatogram of spinach FNR on a barley Fd-Sepharose column. The barley Fd-Sepharose column was applicable to the affinity column chromatography of spinach FNR under the same conditions as established with a spinach Fd-Sepharose column [6], although it was less specific for spinach FNR, as shown in Table I.

Fig. 4 shows a chromatogram of spinach FNR on

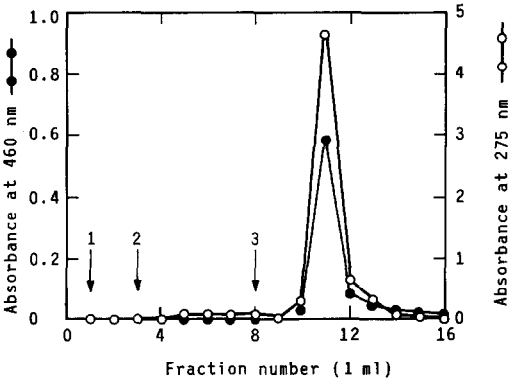

Fig. 3. Affinity chromatography of spinach FNR on a barley Fd-Sepharose column. A barley Fd-Sepharose column (4 × 1 cm I.D.) was equilibrated with 20 m*M* Tris–HCl buffer (pH 7.5). Spinach FNR dissolved in 20 m*M* Tris–HCl buffer (pH 7.5) was loaded at arrow 1. Arrow 2, 20 m*M* Tris–HCl buffer (pH 7.5) was added. Arrow 3, the same buffer containing 0.25 *M* NaCl was added.

a *Synechococcus* Fd-Toyopearl column operated with an automated fast-flow liquid chromatographic system. When a crude preparation of spinach FNR was adsorbed on the column at low ionic strength, a large amount of contaminating proteins passed through. After washing the column, FNR remaining adsorbed on the column was eluted in a sharp peak by increasing the NaCl concentration in

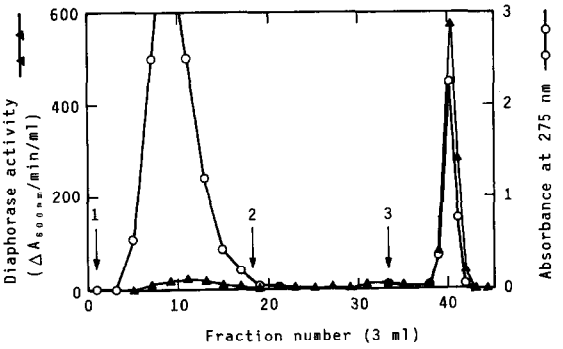

Fig. 4. Affinity chromatography of spinach FNR on a *Synechococcus* Fd-Toyopearl column. *Synechococcus* Fd-Toyopearl was packed in a pressure-resistant glass column (10 × 2.2 cm I.D., C.I.G. type) to a height of 4.1 cm and placed in a fast-flow liquid chromatographic apparatus (Toyopearl Chromato). The Fd column (4.1 × 2.2 cm I.D.) was equilibrated with 20 m*M* Tris–HCl buffer (pH 7.5) at a flow-rate of 1 ml min^{-1}. At arrow 1, the eluent was automatically changed to crude spinach FNR dissolved in the Tris–HCl buffer; arrow 2, to the Tris–HCl buffer; arrow 3, to the Tris–HCl buffer containing 0.25 *M* NaCl.

the eluent to 0.25 *M*. During this affinity chromatography, the absorbance ratio of the FNR preparation, A_{460}/A_{275}, increased from 0.024 to 0.114. The latter value corresponds to a purity of 85.7%, taking the absorbance ratio of a pure spinach FNR as 0.133 [6]. At the same time, the specific activity increased 8.58-fold. The binding capacity of the column remained unchanged during repeated use. All these observations demonstrated that the *Synechococcus* Fd-Toyopearl column is more effectively applicable to purification of FNR than other immobilized ferredoxins on Sepharose.

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